oocyte expression system, Kahle *et al.*[3] now show that WNK4 also inhibits ROMK activity by stimulating clathrin-dependent endocytosis of ROMK, a mechanism distinct from WNK4 inhibition of NCCT. Notably, the mutations in *PRKWNK4* that cause PHAII further stimulate the retrieval of ROMK from the cell surface, which may help explain the impaired potassium secretion and consequent hyperkalemia observed in PHAII. Together, these observations establish the reciprocal effect of mutations in *PRKWNK4* that cause PHAII on the routing of NCCT and ROMK to the plasma membrane. Thus, WNK4 is a novel dual regulator that balances both sodium reabsorption and potassium secretion in the distal part of the nephron, a site where the final composition of the urine is determined.

### A WNK and a nod

In light of the identified actions of wild-type and mutant WNK4, we may now be able to explain at the molecular level the nephron's ability to control independently sodium-chloride reabsorption and potassium secretion in response to varying conditions. Several physiologically important roles can now be envisioned (**Fig. 1**). Aldosterone is a primary regulator of both sodium-chloride reabsorption and potassium secretion. The baseline condition resembles a situation of low aldosterone levels in which WNK4 continuously suppresses the routing of both transporters, resulting in only a modest number of active transport proteins at the plasma membrane. In the presence of substantial levels of aldosterone, two extreme situations can occur. In hypovolemic states (reduced blood volume), the inhibition of NCCT by WNK4 is relieved, whereas the routing of ROMK to the plasma membrane is abolished, allowing maximal sodium-chloride reabsorption. Similarly, during conditions of hyperkalemia, the inhibition of ROMK is lessened, whereas the routing of NCCT is blocked, maximally stimulating potassium secretion. Thus, this model predicts a differentiating role of WNK4 in aldosterone-regulated sodium and potassium homeostasis.

### Intriguing questions

Several outstanding issues can now be addressed experimentally to improve our understanding of the bifunctional role of WNK4 in general and in PHAII in particular. For instance, the subcellular localization of WNK4 to intercellular junctions and cytoplasmic compartments hints at additional, unknown functions along the nephron. The clinical features of PHAII are chloride-dependent. Infusion of sulfate as an anion replacing chloride corrects the elevated levels of potassium in the urine (hyperkaliurea) observed in PHAII (ref. 10). Together with the conspicuous localization of WNK4 in the tight junction complex, this suggests that WNK4 could regulate the paracellular chloride conductance. In addition, the dominant mode of inheritance of PHAII is difficult to reconcile with the experimental data obtained so far, suggesting that the situation is more complex than presently envisioned or that the *X. laevis* oocyte expression system may not reproduce an effect that occurs in the human kidney. Thus, further studies in polarized epithelial cells are certainly warranted to further substantiate the (patho)physiological role of WNK4.

Finally, many details of the mechanism of action of WNK4 have yet to be addressed. WNK4 contains several potential regulatory sites that illuminate the signaling pathways that can now be explored. The catalytic kinase domain in WNK4 indicates that phosphorylation of downstream target proteins could have an important regulatory role. Initial studies indeed suggest that WNK4 inhibits NCCT in a kinase-dependent manner, but the inhibition of ROMK is kinase-independent, suggesting additional pathways[3,8]. Thus, further work is necessary to determine conclusively all the functions of the regulatory domains in WNK4. The outlined studies again illustrate the importance of unraveling the molecular defects of rare genetic diseases, such as PHAII, to establish new concepts in the physiology of renal salt and water homeostasis.


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# Free energy lights the path toward more effective RNAi

Jose M Silva, Ravi Sachidanandam & Gregory J Hannon

**Silencing gene expression by RNA interference (RNAi) has become a daily activity for mammalian cell biologists. Those who often use this approach have come to appreciate its occasional frustrations and potential limitations. A pair of recent papers in *Cell* provides rules for designing short interfering RNAs (siRNAs) that are both validated at the practical level and understood mechanistically.**

The discovery that double-stranded RNA can elicit RNAi, a specific, potent gene silencing response, began a genetic revolution in those organisms for which genetic approaches were largely unavailable or inconvenient. Generally, the interference process initiates with the processing of a double-stranded RNA by Dicer (reviewed in ref. 1). This RNaseIII-class enzyme creates siRNAs, which comprise duplexes of ~19 nucleotides (nt) bounded by 2-nt 3′ overhangs. The siRNAs become incorporated into the RNA-induced silencing complex (RISC), which uses such sequences as guides to the selection of its substrates by Watson-Crick base-pairing interactions. Thus, siRNAs must be unwound for exposure to potential targets, which is thought to be mediated by an ATP-dependent RNA helicase[2]. Single-stranded versions of siRNAs can be used to trigger RISC assembly *in vitro* in both *Drosophila melanogaster* and mammalian cell extracts, although they are less efficient triggers than double-stranded siRNAs[3,4].

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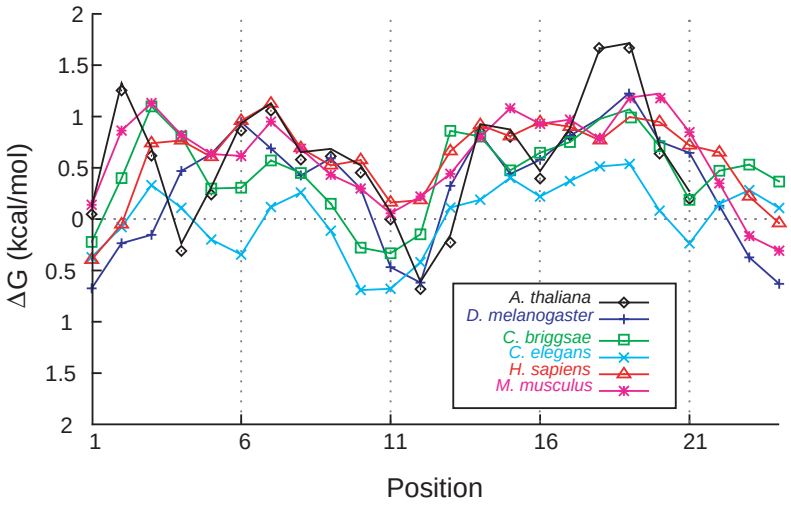


**Figure 1** Doing it the natural way. Stability profiles were calculated according to Turner using a nearest neighbor approach with gap penalties (http://www.bioinfo.rpi.edu/~zukerm/rna/energy/; see, for example, ref. 10) for miRNAs available in the Sanger RFAM database[11]. Numerical values differ from those in ref. 7 because in this case, average stabilities are calculated for each individual position rather than as a scanning average for pentamers. miRNAs were aligned according to the 5′ end of the mature sequence (aligned at position 1 in the plot). Data is presented for human (*Homo sapiens*; 143 sequences), mouse (*Mus musculus*; 61 sequences), *Caenorhabditis elegans* (101 sequences), *Caenorhabditis briggsae* (49 sequences), *D. melanogaster* (64 sequences) and *Arabidopsis thaliana* (25 sequences). Variants of miRNAs (*e.g.*, multiple let-7 family members) were excluded to avoid biasing the analysis. Because miRNAs vary in length, fewer data point were used, and thus less statistically significant values were obtained, for positions past 20.

Tuschl and colleagues[5] and Caplen and coworkers[6] first found that siRNAs themselves could be used to trigger RNAi responses in mammalian cells. In practice, the approach can be made to work well, achieving reduction of target mRNAs by >90% after transfection of cells with synthetic siRNAs. But achieving this goal often requires screening many siRNAs before truly effective inhibitors are discovered. Two papers published recently in *Cell* promise to improve the success rate of the approach through the discovery of real, mechanism-based guidelines for siRNA selection[7,8].

## Eliminate free energy!

Zamore and colleagues arrived at their insights into siRNA design by solving a mystery that emerged from their ongoing *in vitro* studies of RISC biochemistry[8]. They first compared single-stranded and double-stranded siRNAs and their respective abilities to direct the cleavage of either sense- or antisense-oriented luciferase target RNAs. Both strands of the siRNA worked equally well when presented to the extract as single strands. With double-stranded molecules, however, there was profound strand bias in ability to direct target cleavage. Thus, the two strands did not differ significantly in their intrinsic efficacy but the ability of individual strands to silence, *in vitro*, differed if the strands were presented in double-stranded form. In a novel RISC-capture assay, strand asymmetry was apparent, with the antisense strand (with respect to the coding orientation of the luciferase mRNA) showing both better incorporation into RISC and more efficient cleavage for this particular siRNA.

The authors' examination of the sequence of the siRNA identified one immediately obvious difference between the two termini of this molecule. The 5′ end of the strand that was incorporated into RISC more efficiently had a lower stability and began with an A-U pair, whereas the less effectively incorporated strand had a G-C terminus. This led to the hypothesis that RISC preferentially accepts the strand of the siRNA that presents the less stable 5′ end. To test this notion, Zamore and colleagues made numerous alterations to a series of siRNAs that target *sod1 in vitro*. By altering the free energy of base pairing at the ends (by creating a mismatch or just changing the bases), and also throughout the neighboring three nucleotides by mismatches, the effective strand of the siRNA could be manipulated in a predictable manner.

MicroRNAs (miRNAs) are small, endogenously encoded hairpins that can regulate the expression of target mRNAs (reviewed in ref. 1). Current working models suggest that these are processed by Dicer and enter RISCs that are either closely related to or identical to those occupied by siRNAs. One mystery that has surrounded miRNAs since their discovery was that only one strand of the miRNA was generally detected by cloning or northern blotting. When Zamore and colleagues mapped their conclusions from the study of asymmetric siRNAs onto *D. melanogaster* miRNAs, they discovered that the instability of the 5′ end of the mature miRNA could largely predict its preferential incorporation into a RISC. A similar analysis is recapped in **Figure 1**. This hypothesis provides a logical solution to the problem of miRNA asymmetry that fits the observed biochemistry much better than previous models, which depended on strand-specific Dicer activity.

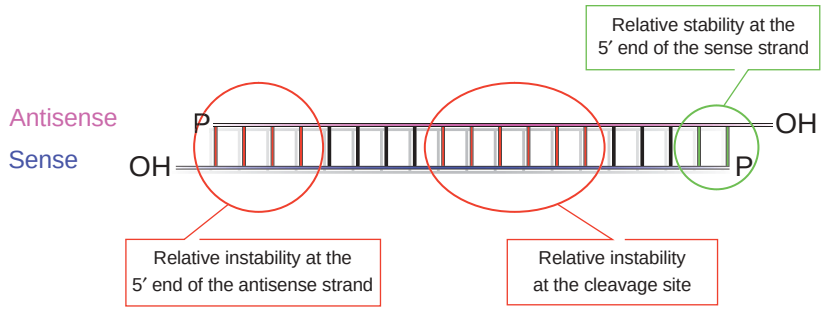


**Figure 2** Habits of highly effective siRNAs. Insights into siRNA design are depicted schematically.

## Cells toe the line

Jayasena and colleagues arrived at similar conclusions but approached the problem from a different angle[7]. With a practical interest in producing more effective siRNAs, they began with an analysis of naturally occurring miRNAs. These triggers were predicted to have been evolutionarily

selected for the ability to regulate targets effectively. These investigators noted that the average stability of the miRNA was not random along its length (**Fig. 1**). Mature miRNAs were unstable at their 5′ ends. Internal stability was also lower near the center of the miRNA (around positions 9–11). Notably, an examination of 37 siRNAs targeting several different genes showed that effective sequences (16 of 37) matched the miRNA-derived patterns whereas ineffective sequences (21 of 37) did not. They obtained additional consistent data with a wider panel of 180 siRNAs targeting a region of firefly luciferase.

The bias in strand-specific cleavage by RISC was not tested *in vivo*. But siRNAs matching the predicted profiles accumulated preferentially from an expressed, long double-stranded RNA trigger in tobacco. This observation matches the proposal that strand-specific incorporation into RISC determines both the stability and, to a large degree, the efficacy of siRNAs, irrespective of whether they are synthetic RNAs that are experimentally introduced or are generated by Dicer *in vivo*.

The combination of *in vitro* and *in vivo* studies provide complementary insights into the design of effective siRNAs (summarized in **Fig. 2**). These insights will probably also guide the design of more effective expressed silencing triggers, which enter the RNAi pathway by mimicking miRNAs. Of course, this achievement will depend on a detailed understanding of how synthetic miRNAs give rise to specific siRNA sequences *in vivo* through the actions of Dicer and Drosha[9]. Finally, the studies highlighted here shed light on the mechanisms of RISC assembly, suggesting that inbuilt structural cues create specificity through asymmetry in what might superficially appear as a symmetric siRNA. In the final tally, the reports reviewed here make a clear case that with RNAi, the best way to win the game is to play by the rules.

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